Effect of α7 nicotinic acetylcholine receptor activation on cardiac fibroblasts: A mechanism underlying RV fibrosis associated with cigarette smoke exposure.

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Abstract:

Introduction: Right ventricular dysfunction is associated with numerous smoking-related illnesses including chronic obstructive pulmonary disease (COPD) where it is present even in absence of pulmonary hypertension. It is unknown if exposure to cigarette smoke has direct effects on RV function and cardiac fibroblast proliferation or collagen synthesis. In this study, we evaluated cardiac function and fibrosis in mice exposed to cigarette smoke (CS) and determined mechanisms of smoke-induced changes in cardiac fibroblast signaling and fibrosis.

Methods: AKR mice were exposed to 6 weeks cigarette smoke followed by echocardiography and evaluation of cardiac hypertrophy, collagen content, and pulmonary muscularization at study conclusion. Proliferation and collagen content were evaluated in primary isolated rat cardiac fibroblasts (CF) exposed to cigarette smoke extract (CSE) or nicotine. Markers of proliferation, fibrosis, and proliferative signaling were determined by immunoblot or Sircol collagen assay.

Results: Mice exposed to CS had significantly decreased RV function as determined by TAPSE. There were no changes in LV parameters. RV collagen content was significantly elevated but there was no change in RV hypertrophy or pulmonary vascular muscularization. CSE directly increased cardiac fibroblast proliferation and collagen content in CF. Nicotine alone reproduced these effects. CSE and nicotine-induced fibroblast proliferation and collagen content was mediated through α7 nicotinic acetylcholine receptors and was dependent on PKC-α, PKC-δ, and reduced p38-MAPK phosphorylation.

Conclusion: CS and nicotine has direct effects on cardiac fibroblasts to induce proliferation and fibrosis which may negatively affect right heart function.

Introduction

Right ventricular dysfunction is a poor prognostic marker in a number of cardiopulmonary diseases such as heart failure, pulmonary hypertension, and chronic obstructive pulmonary disease (COPD). Cigarette smoking is a major cause of COPD. RV dysfunction in smokers is thought to be precipitated by vascular remodeling associated with COPD resulting in pulmonary hypertension and subsequently increased RV afterload (12, 22). However, recent evidence has shown that RV dysfunction can be present in COPD patients without pulmonary hypertension (12). RV dysfunction and failure is associated with increased RV fibrosis (2), demonstrated in autopsy specimens and in animal models of RV failure (2, 11). Myocardial fibrosis is mediated by proliferation and activation of cardiac fibroblasts. However, it is unknown if exposure to cigarette smoke has direct effects on RV function, cardiac fibroblast proliferation, or collagen synthesis.

Cigarette smoke is a complicated chemical mixture. The effects of cigarette smoke on cellular function have been attributed to various components such as nicotine, reactive oxygen species (ROS), and reactive aldehydes, amongst others. In endothelial cells, exposure to CSE induces apoptosis via reactive oxygen species and reactive aldehydes (6, 24). CSE can also promote increased apoptosis of cardiomyocytes (7, 34). In contrast, in cancer cells, nicotine may promote tumor cell proliferation and survival (25). Furthermore, nicotine is fibrogenic in multiple organs including the lungs and kidneys (13). However, the effect of exposure of cigarette smoke constituents on ventricular fibroblasts and the underlying mechanism(s) remain unclear.

In this study, we sought to evaluate the effect of cigarette smoke exposure on right ventricular function. Furthermore, we sought to determine the effects of cigarette smoke on primary cardiac fibroblast proliferation and elucidate the underlying mediator and the mechanism. We demonstrate that CS smoke exposure in mice increases RV collagen content and significantly reduces RV function in

the absence of RV hypertrophy and vascular remodeling. In addition, nicotine exposure recapitulates the effect of CS on cardiac fibroblasts causing increased fibroblast proliferation and collagen content mediated via α7 nicotinic acetylcholine receptor (nAChR) activation.

Materials and Methods:

Materials. All materials were obtained from Sigma (St. Louis, MO) unless otherwise noted. Collagenase II and DNAse were purchased from Worthington (Lakewood, NJ). α-Smooth muscle actin (α-SMA, ab7817) antibody and α-Bungarotoxin (α-BTX) were purchased from Abcam (Cambridge, MA). Alda-1 was purchased from EMD Millipore (Billerica, MA). The vectors encoding dominant negative cDNA for PKC-δ (pHACE-PKCδ^{K376R}) and PKC-α (pHACE-PKCα^{K368R}) were gifts from Bernard Weinstein (Addgene plasmids #16389 and #21235, respectively, Cambridge, MA(27)) . cDNA for green fluorescent protein (pGFP-C1) was obtained from Clontech (Mountain View, CA). Sircol collagen assay was purchased through Accurate (Westbury, NY). Antibodies against phosphorylated Erk (T^{202}/Y^{204}, #9101), phosphorylated p38 (T^{180}/Y^{182}, #9125), phosphorylated Akt (T^{308}, #9272), total Erk (#9102), total p38 (#9212), and total Akt (#9272) were purchased through Cell Signaling (Beverly, MA). α7 nAChR siRNA (sc-270402), control siRNA-FITC (sc-36869), antibodies for HA (sc-805), PKC-α (sc-208), PKC-δ (sc-8402), PKC-βII (sc-210), Procollagen (sc-8787), α7 nAChR (sc-554), caspase-3 (9662) and PCNA (sc-25280) were purchased from Santa Cruz Biotechnology (Dallas, TX). von Willebrand Factor antibody (#2022-04) was purchased from Dako (Carpinteria, CA). Lipofectamine3000 was purchased through Life Technologies (Grand Island, NY).

Animals. All animal protocols were approved by the Institutional Animal Care and Use Committee of the Providence VA Medical Center and comply with the Health Research Extension Act, US Public Health Service, and US National Institutes of Health Policy (protocols 2010-026 and 2013-010). Male AKR mice ages 6-8 weeks purchased from Jackson Laboratory were either exposed to room air (RA) or cigarette smoke (CS) for six weeks at 6 hours per day, 4 days a week using a TE-10 mouse smoking machine (Teague Enterprises, Woodland, CA) with 3R4F reference cigarettes (University of Kentucky, Tobacco Research Institute, Lexington, KY) as previously described (24). The smoking machine produced a mixture of 89% sidestream and 11% mainsteam smoke and the chamber was monitored for 120 mg/m^3 of total suspended

particulate matter. After 6 weeks, mice were weighed, and were subjected to either transthoracic echocardiography or in vivo hemodynamic measurements and then euthanized. These studies were performed in two cohorts with the all animals in the first groups receiving echocardiography and all animals in the second group subjected to invasive hemodynamics. Lung and heart tissue were collected. Lungs were fixed with 10% formalin at 25 cm of H_2O pressure, paraffin embedded, and sectioned for α-SMA/vWF staining. Hearts were fixed in formalin, paraffin embedded, and sectioned for lectin and picrosirius staining.

Adult Sprague-Dawley rats 250-300 g purchased from Charles River (Wilmington, MA) were used for cardiac fibroblast isolations (see below).

Echocardiographic Measurements. A subset of animals were anesthetized with continuous isoflurane inhalation (1.5-3%) and transthoracic echocardiography was performed as described (5). A 40 MHz linear array transducer was used (Vevo 2100, VisualSonics, Toronto, ON, Canada). Two-dimensional, Doppler, and M-mode recordings were obtained to measure LV fractional shortening and LV and RV dimensions. The pulsed-wave Doppler recording at the right ventricular overflow tract was used to measure pulmonary acceleration time (PAT). Tricuspid annular plane systolic excursion (TAPSE) was measured by use of M-mode across the tricuspid valve annulus at the RV free wall. TAPSE was determined by measuring the excursion of the tricuspid annulus from its highest position to the peak descent during ventricular systole. Tissue Doppler was used to measure the early diastolic velocity of the septum (at mitral annulus) and RV lateral wall (at tricuspid annulus).

In Vivo Hemodynamics. RV systolic pressure (RVSP), LV systolic pressure (LVSP), and pulmonary artery systolic pressure (PASP) were measured under isoflurane anesthesia (1.5-2%) using an opened-chest technique. After the chest is opened, a high-fidelity pressure-volume 1.0F catheter was inserted into a puncture to apex of the LV and pressure measurements were recorded for 15 sec. Subsequently, the same

catheter was inserted into the apex of the RV and pressure measurements were recorded for another 15 sec. The catheter was then guided into the pulmonary artery just past the pulmonic valve to record the PAP for 15 sec. Mean PAP (MPAP) was obtained by utilizing the equation: (PASP – 2PADP)/3, where PADP is the PA Diastolic Pressure. The animal was then euthanized and tissue was collected for further analysis.

Pulmonary Muscularization. Paraffin embedded lung tissue was sectioned and stained for α-smooth muscle actin and von-Willebrand factor (vWF) as previously described (4). Tissue sections were then scanned into an Aperio CS slide scanner at 20x magnification. 50 equally sized fields were marked onto the tissue image and scored for colocalized staining of α-SMA and vWF. Scoring was categorized as follows: "none" if no α-SMA staining colocalized with vWF, "partial" if less than 50% α-SMA staining colocalized with vWF, and "complete" muscularization if greater than 50% α-SMA staining colocalized with vWF.

Picrosirius Red staining and Coronary Vessel Counts: Paraffin-embedded heart tissue was sectioned and deparaffinized. Picrosirius stained slides were imaged using a Nikon Eclipse-NI microscope with 20X objective, images were taken in the red (594 nm) and green fluorescent (488 nm) channels as well as brightfield. Three images each were taken from the RV, LV, and septum from each heart. Picrosirius Red fluoresces in the red channel and quenches green autofluorescence . Therefore, analysis of picrosirius red positive stained area was performed by subtraction of the green fluorescence signal from the red fluorescence signal (Nikon Image Software - Elements) similar to previously published protocols (31). ImageJ was then used to threshold the subtracted fluorescent Picrosirius red stained image. Thresholded images were used for % area quantiation and the data was normalized to the respective RA control. For coronary microvessel analysis antigen retrieval was performed with boiling 10 mM sodium citrate and 0.05% Tween 20, pH 6.0 for 10 min. Sections were then blocked with 5% BSA in PBS and incubated overnight with Isolectin GS-IB4, Alexa Fluor-488 Conjugate (Thermo Scientific #I21411), in

2.5% BSA at 4°C. Sections were then mounted with coverslips using Prolong Gold with DAPI imaged with a fluorescent Nikon Eclipse E400 microscope. Equal-size areas were then counted manually for positive staining of lectins for microvascular vessels at 20X magnification. Averaged vessel counts were normalized to mm^2.

Collagen Content Measurement. Collagen content was measured using Sircol collagen dye binding assay per manufacturer's instructions. Equal amounts of protein or homogenates were mixed with equal volume of Sircol dye reagent for 30 min with agitation. The collagen-dye complex was centrifuged at 12,000 rpm for 10 min and unbound dye was aspirated. The remaining pellet was washed with ice cold acid-salt wash reagent (acetic acid, sodium chloride, and surfactants). Samples were then centrifuged again at 12,000 rpm for 10 min. The wash reagent was aspirated and the remaining pellet was dissolved by adding the alkali reagent (0.5 M sodium hydroxide). After 5 min incubation, collagen content was measured using absorbance at 555 nm.

Cardiac Fibroblast Isolation. Adult rat cardiac fibroblasts were isolated as previously described (35). Briefly, adult Sprague-Dawley rats (250-300 g) were euthanized with continuous inhalation of 5.0% isoflurane. Hearts were immediately excised, trimmed of extra-aortic tissue, and retrogradely perfused for 2 min in Krebs-Henseleit (KH) Buffer at 37°C and then switched to *enzyme buffer 1* (KH buffer containing 0.3 mg/mL collagenase II, 0.3 mg/mL hyaluronidase, and 50 µM $CaCl_2$) for 18 mins. After perfusion, the ventricular tissue was excised and in some cases as indicated in results (Figure 4G) was divided into LV and RV tissue. Myocardial tissue was minced with scissors and further digested in *enzyme buffer 2* (enzyme buffer 1 supplemented with trypsin IX 0.6 mg/mL, deoxyribonuclease 0.6 mg/mL, and increased $CaCl_2$ to 500 µM) at 37°C for 18 min in a shaking waterbath. The digestion was stopped with the addition of 10 mL of DMEM supplemented with 10% FBS, penicillin, and streptomycin (*complete media*), filtered through a 200 µm nylon mesh, and centrifuged at 500 rpm for 5 min. The supernatant was

removed and centrifuged again at 2000 rpm for 5 min. The resulting pellet was resuspended in *complete media* and plated into four 10 cm dishes. The media was changed after two hours to remove cellular debris and unbound cells. Fibroblast purity was at least 95% after plating for 24 hours and washing with media. This method previously demonstrated a fibroblast population which stained positive for vimentin and minimal amounts of vWF and smooth muscle myosin and actin in low passage cells(P0-P2) (35). Cells were allowed to grow to confluency within 2-3 days before being passaged for in vitro experiments. Only Passage 1 (P1) cells were used in this study.

Myocyte Contractility. The mechanical properties of the cardiomyocytes were assessed using an IonOptix Myocam System (IonOptix Inc., Milton, MA) as described previously(14). Unloaded cardiomyocytes isolated from each group of mice were placed on a glass slide and allowed to adhere for 5 min, then imaged with an inverted microscope and perfused with Tyrode's buffer at 37 °C. Cardiomyocytes were paced with 10 V, 4 ms squarewave pulses at 1.0 Hz, and data presented as percent length change.

Cigarette Smoke Extract. Cigarette smoke extract (CSE) was prepared as previously described (18). Mainstream smoke from 3R4F cigarettes was drawn into 30 mL of PBS by vacuum. Each cigarette was lit for 5 mins with a total of five cigarettes being used. This was considered 100% CSE. Control solution was prepared by drawing unlit cigarettes into PBS. CSE and control solutions were used immediately. Final concentrations of CSE are expressed as percent values (the ratio of CSE to total medium volume). The pH and color of the CSE was noted after use to maintain consistency.

In vitro Experiments. Subconfluent (60-70%) P1 cells were exposed to serum-free DMEM supplemented with 10 µg/mL insulin, 5.5 µg/mL transferrin, and 5 ng/mL sodium selenite (ITS, Corning, Corning, NY) and penicillin and streptomycin. After 24 hr, cells were then pretreated with inhibitor for 30 min and then treated with either PBS (CSE vehicle)/CSE or ethanol (nicotine vehicle)/nicotine (600 nM or unless otherwise noted) for another 24 hr. For proliferation assays, cells were trypsinized and counted with a

hemocytometer. For cell viability assays, cells were loaded with the tetrazolium dye MTT for 2-4 hr. Media was removed and cells were washed with PBS. MTT detergent (isopropanol/HCl) was then added and absorbance was read at 590 nm after an hour with a multiplate reader (BioTek).

Western Immunoblotting. RV and LVS from mice were homogenized at 4°C in homogenization buffer (20 mM HEPES, 250 mM sucrose, 100 mM NaCl, 0.2 mM EDTA, 0.2 mM EGTA, 200 µM PMSF, 0.5 mM DTT, 1 µM leupeptin, 1 µM aprotinin, and phosphatase inhibitor cocktail III). Homogenates were then centrifuged at 5,000 rpm for 10 min at 4°C. The pellet was discarded and supernatant was then centrifuged again at 15,000 rpm for 10 mins at 4°C. Supernatants were used for total protein analysis. Cardiac fibroblasts from the in vitro studies were collected in radioimmunoprecipitation assay buffer (RIPA) as previously described (29) and incubated on ice for 10 min prior to centrifugation for 10 min at 15,000 rpm. Protein concentration was determined using Bradford-Lowry Assay (Biorad). Proteins (50 µg/lane) were resolved on 7.5% (procollagen) and 10% (Erk, PKCs, PCNA, HA, Akt, p38) separating gels by SDS-PAGE. Resolved proteins were transferred to polyvinylidene fluoride membrane and immunoblot analysis was performed by appropriate antibody dilution of 1:1,000 for all antibodies with the exception of procollagen and HA (1:200). Blots were then incubated with the proper HRP-conjugated secondary (SCBT) for 1 hr at RT and chemiluminescence generated with Bio-Rad Clarity. Blots were imaged with a film developer or GE Healthcare ImageQuant. Equal loading was confirmed by probing for vinculin (1:3,000) or actin (1:1,000). Quantitative densitometry was performed by use of the public domain ImageJ program.

cDNA and siRNA transfections in Cardiac Fibroblasts. Cardiac fibroblasts were transiently transfected with cDNA (4 µg/well of six-well plate) encoding dominant negative PKC-δ (PKC-δ^{K376R}), PKC-α (PKC-α^{K368R}), or GFP and α7 siRNA (600 nM) or scrambled siRNA (600 nM) using Lipofectamine3000 according

to manufacturer's instructions. After 24 hrs post-transfection, cells were quiesced for 24 hrs and then used for experiments as described.

Statistical Analysis

Statistical tests were either unpaired 2-tailed Student's t-test, One Way ANOVA with Dunnets post-hoc test, or Two Way ANOVA with Tukey post-hoc test as appropriate and unless otherwise indicated. All data are expressed as mean ± S.E.M unless otherwise indicated. A p-value < 0.05 was considered statistically significant.

RESULTS:

CS exposure is associated with RV dysfunction and fibrosis in absence of right ventricular hypertrophy and pulmonary vascular remodeling: Exposure to 6 weeks of CS significantly decreased RV function as assessed by TAPSE. The TV e' and E/A ratio were also reduced suggesting the presence of RV diastolic dysfunction (Table 1, Figure 1A). There were no significant changes in RV or LV pressures or pulmonary artery pressures as assessed by invasive hemodynamics with a pressure catheter (Figure 1B) or estimation of PA pressure assessed via pulmonary acceleration time (PAT) (Table 1). There were no changes in systemic blood pressure, heart rate, or other indices of left ventricle systolic or diastolic function (Table 1, Fig 1). There were also no changes following only 3 weeks of exposure to CS (data not shown). In parallel to the changes in RV function, RV collagen content as assessed by Sircol Assay was increased in the CS exposed animals at 6 weeks (Figure 2A) but not at 3 weeks (data not shown). This was confirmed by increased procollagen expression (Figure 2B) in the RV of CS exposed mice compared to controls. In contrast, there was no increase in collagen content or procollagen expression in the left ventricle (Figure 2A, B). Collagen content of only septal tissue was also evaluated using Sircol assay and no differences were found with CS exposure (data not shown). Collagen content and fibrosis were also evaluated using picrosirius red staining (Figure 2C). Increased levels of collagen staining were apparent in the RV of CS exposed mice compared to room air (Figure 2C). Quantitation of picrosirius staining demonstrated significant increases only in the RV of CS exposed mice, similar to Sircol Assay (Figure 2D) Although collagen content was increased in the RV, there was no gross right or left ventricular hypertrophy (Table 2) between groups as determined by tissue weight. CS treated animals had overall reductions in both body and tissue weights (Table 2); however RV and LV+S/BW ratios remained unchanged. There were also no changes in whole heart expression or cleavage of caspase-3 in the RV or LV of either CS or RA animals indicating enhanced apoptosis did not contribute to reduced RV function (data not shown). In addition, there was no notable presence of coronary microvascular rarefaction as determined by vessel counts in

CS treated animals (Table 2). There was some evidence of mild air-space disease in CS-smoked animals as shown by an increase in alveolar airspace represented by mean linear intercept measured in H&E lung cross sections (Table 3). However, evaluation of pulmonary vascular histology did not show any smoke-induced changes in muscularization of small, medium, or large sized vessels compared to control (Table 2). Therefore, CS may induce direct effects on RV function and remodeling independent of significant changes in afterload, LV dysfunction, and lung vascular remodeling.

Cigarette smoke extract induced cardiac fibroblast proliferation: The effects of cigarette smoke extract (CSE) on fibroblast proliferation were assessed in adult rat cardiac fibroblasts isolated from whole hearts (both RV and LV). Interestingly, increasing concentration of CSE resulted in CF proliferation as assessed by cell counts (Figure 3A) and cell viability as assessed by MTT assay (Figure 3B) in isolated fibroblasts. There was also an increase in the proliferative marker, PCNA (Figure 3C). There were no changes in the ratio of cleaved to total caspase-3 indicating effects were not due to reduced apoptosis (data not shown).

CSE contains the reactive aldehyde acrolein and reactive oxygen species (ROS) which may underlie some of the cardiac effects of CS exposure. Pretreatment of CF with Alda-1, an aldehyde dehydrogenase activator, to reduce reactive aldehydes and ROS scavenging with N-acetyl cysteine did not inhibit CSE-induced CF proliferation (Figure 3D). Lower doses (100 nM) of both Alda1 and NAC also had no effect (data not shown). In contrast, pretreatment with mecamylamine (Mec), a general nicotinic acetylcholine receptor (nAChR) antagonist, attenuated the effect of CSE on CF proliferation (Figure 3E).

***Activation of α7 nAChR mediated CF proliferation*.** Since effects of CSE were mediated via activation of nAChRs, we examined if nicotine was sufficient to promote cardiac fibroblast proliferation and increase in collagen through nAChR. Similar to CSE, nicotine increased CF proliferation (Figure 4A). Nicotine also increased collagen content in a dose-dependent manner (Figure 4 B). 600 nM nicotine also increased procollagen expression (Figure 4C). The effective doses of nicotine to induce proliferation were similar to

those previously measured in the blood of smokers (25-444 nM) (23). Nicotine-induced proliferation was blocked by pretreatment with mecamylamine (Figure 4A and B). Similarly, the effect of nicotine was blocked by α-bungarotoxin, a specific α7 nAChR antagonist (Figure 4D). Knockdown of α7 nAChR receptors with siRNA (Figure 4E) also blocked nicotine-induced proliferation (Figure 4F). To investigate one potential cause of RV selective CS-induced fibrosis, α7 nAChR expression was next examined in RV and LV tissue. There were no changes in the expression of α7 nAChR (Figure 4G). As fibroblasts used in all the previous experiments were from mixed RV and LV populations, we isolated fibroblasts specifically from the LV and RV of male and female mice. We found no difference in nicotine-induced proliferation in fibroblasts isolated from LV or RV of male or female rats (Figure 4H) (Three Way ANOVA, effect of nicotine p-value < 0.01, effect of chamber p-value = 0.85, and effect of sex p-value = 0.89, there were no significant interactions). The consistent response to nicotine in fibroblasts isolated from whole hearts or specific chambers indicates that there are additional physiologic factors or possibly other constituents in CS that may positively (RV) or negatively (LV) modulate fibroblast proliferation *in vivo* following CS exposure. In addition, acute nicotine treatment (1 hour) did not cause any direct effects on LV or RV cardiomyocyte contractile function (Figure 4I), further supporting the concept that nicotine-induced fibroblast proliferation is responsible for impacting cardiac function.

Nicotine-induced fibroblast proliferation is PKC-α and -δ– dependent. CFs were pretreated with either Gö6976, LY333531, or Rottlerin to inhibit classical PKCs, PKC-β, or PKC-δ, respectively (Figure 5A-C). Inhibition of PKC-α/β with Gö6976 (Figure 5A) attenuated nicotine-induced CF proliferation. This effect was absent using the PKC-β specific inhibitor LY333531 alone (Figure 5B), indicating effects of Gö6976 were attributable to inhibition of PKC-α. PKC-δ inhibition (Figure 5C) with rottlerin also attenuated nicotine-mediated CF proliferation. Transfection of CF with dominant negative plasmids of PKC-α and -δ (Figure 5D-E) also blunted nicotine-induced CF proliferation confirming results with small molecule inhibitors. Gö6976 and rottlerin also blocked nicotine-induced increase in collagen content (Figure 5F).

In addition, immunoblots of RV lysates from smoke exposed mice confirmed a role for PKC signaling *in vivo* with increased expression of PKC-α and -δ compared to RA controls (Figure 5G,H). We did not detect any change in PKC expression in the LV (data not shown).

Nicotine reduces p38 MAPK activation but does not promote other common proliferation signals. We next evaluated nicotine-induced changes in downstream pathways related to acute nAChR signaling including Erk, Akt, and p38-MAPK (Figure 6A). While no changes were noted in phosphorylation of Erk or Akt (Figure 6A), nicotine decreased p38-MAPK phosphorylation after 30 min nicotine exposure (Figure 6A, B). Decreased p38 phosphorylation in response to nicotine was attenuated by pretreatment with the general nAChR antagonist mecamylamine (Figure 6C).

Nicotine-reduced p38-MAPK activation is mediated by PKC-δ but not PKC-α. Pretreatment of rat cardiac fibroblasts with the PKC-α/β inhibitor Gö6976 did not block nicotine-induced reductions in p38-MAPK activity (Figure 7A and B). In contrast, treatment of cells with rottlerin significantly attenuated nicotine-induced reductions in p38-MAPK activity (Figure 7C and D).

Discussion:

The principle findings of this study were the following: 1) that 6 week cigarette smoke exposure induced RV dysfunction and fibrosis in mice independent of overt pulmonary remodeling, 2) CS-induced pro-fibrotic changes were not found in left ventricular tissue, and 3) nicotine directly induce cardiac fibroblast proliferation and collagen content through α7 nAChR dependent PKC-α and -δ signaling.

Our results indicate that cigarette smoke directly changes RV function and collagen content. Importantly, depressed RV contractile function and enhanced fibrosis observed in this study appeared to be independent of remodeling of the pulmonary circulation and increased afterload (as determined by PAT, histological examination, and unchanged PA and RV pressures). In addition, we believe that nicotine is likely the causative factor of increased RV fibrosis since nicotine application to cardiac fibroblasts increased cell proliferation and collagen content. The effects of nicotine on proliferation are cell and tissue dependent with enhanced proliferation in certain cancers and increased apoptosis in endothelial cells (6, 24, 25). In contrast to endothelial cells, nicotine is known to directly increase proliferation in numerous cell types, potentially through activation of nAChR (8, 10, 13). Multiple fibroblast types express α7 nAChR (1, 13). To our knowledge, this is the first study demonstrating direct effects of nicotine on cardiac ventricular fibroblasts mediated via nAChR. General or specific blockade of α7 nAChR with mecamylamine and α-BTX, respectively, or specific knockdown of α7 nAChR was capable of blocking the CSE/nicotine-induced cardiac fibroblast proliferation and profibrotic phenotype.

Importantly, in the CS-exposed mice, enhanced RV fibrosis and depressed function were not associated with an increase in gross RV hypertrophy as assessed by RV/BW ratios. This further indicates that the enhanced RV fibrosis and fibroblast proliferation stimulated by CS was independent of pulmonary vascular remodeling as increased pulmonary pressures and elevated RV afterload would likely be associated with RV hypertrophy and failure as seen in models of COPD and pulmonary

hypertension (30). CS-induced effects were likely mediated by direct nicotine stimulation of fibroblast α7 nAChR. This was supported by direct stimulation of cardiac fibroblast proliferation and collagen expression by nicotine *in vitro*. In addition, to our knowledge, this is the first report of increased RV fibrosis and dysfunction in the absence of simultaneous RV hypertrophy. In this study, we used AKR mice which are prone to CS-induced pulmonary injury. There is no data on use of AKR mice to study pulmonary vascular remodeling and right ventricular function that we are aware of to compare with our model. Others have reported that with certain strains of mice (e.g. A/J) after 5 months of CS exposure, significant vascular remodeling and right ventricular hypertrophy is present. We believe that our model may provide a unique insight into development of RV dysfunction in absence of pulmonary hypertension in settings of CS exposure as noted in patients with COPD and normal PA pressures(26). Further studies are needed to explore the temporal aspect of progression of air space disease, vascular remodeling and right ventricular dysfunction to precisely characterize these relationships in our model of CS exposure.

Another important finding of these studies was that depressed cardiac function and fibrotic signals in response to cigarette smoke were unique to the RV. It is currently unclear why depressed function and fibrotic responses were limited to the RV in CS-exposed mice. Numerous differences have been described between right and left ventricle cardiomyocytes as well as whole heart responses to different agonists and stimuli (9, 22). However, no studies have documented differences in fibroblast specific responses in the two ventricles. Nevertheless, our data clearly indicates that CS induces RV fibrosis and RV dysfunction. Importantly, in our isolated cell experiments, fibroblasts isolated from either the RV or LV had similar response to nicotine. In numerous disease states, the RV is considerably more prone to fibrosis and changes in afterload than the LV (9, 19, 21). Potential explanations for differences in LV and RV fibrosis may be that fibroblast proliferative responses are dependent on chamber specific hemodynamic stress *in vivo* possibly related to air space disease, localized neurohumoral or immune differences, and any potential up or downregulated systems that interact

with nAChR signaling. It is also possible that species specific differences in cardiac fibroblast signaling in response to nicotine exist between the mice hearts and isolated rat CFs used in this study. Finally, crosstalk between cardiac fibroblasts and cardiomyocytes play an important role in modulating cardiomyocyte structure and function(15, 28). While nicotine does not have any direct effect on cardiomyocyte function, it is possible that the nicotine exposed cardiac fibroblasts may modulate cardiomyocyte structure and function contributing to the right ventricular dysfunction observed in cigarette smoke exposed animals. Future studies will be needed to differentiate these possibilities and the important differences observed in LV and RV fibrotic responses in response to CS.

Nicotine-dependent CF proliferation and collagen expression was dependent on α7 nAChR signaling through PKCs. α7 nAChRs are ligand gated ion channels which regulate Ca^{2+} influx in response to acetylcholine or nicotine (1, 8, 13). This study supports the idea that nicotine in CS activates α7 nAChR-mediated Ca^{2+} influx leading to specific PKC isoform activation and subsequent proliferation and fibrosis. The nAChR-mediated Ca^{2+} influx has been associated with activation of Ca^{2+} dependent kinases to mediate downstream proliferative effects (1). We found that nicotine-induced CF proliferation required the Ca^{2+}-dependent PKC-α but did not require the similarly regulated PKC-βII. In addition, the novel PKC isoform PKC-δ was also required for nicotine-induced CF proliferation (Figure 5). Upregulation of PKCs has been implicated in the pathogenesis of LV heart failure and fibrosis (17, 20) and our data indicate that similar PKC signals may be present during cigarette smoke and nicotine-induced RV dysfunction.

Nicotine-induced increased CF proliferation and collagen expression were associated with decreased p38-MAPK phosphorylation which was PKC-δ dependent. The literature regarding p38-MAPK and fibrosis is conflicting; however multiple studies support the notion that decreasing p38 activity in cardiac fibroblasts is pro-fibrotic. In a hypoxia-induced model of pulmonary hypertension (PAH) in rats,

we showed that PAH-induced RV fibrosis is associated with increased RV expression of PKC-βII and PKC-δ (5). RV fibrosis in this model was also associated with decreased p38-MAPK phosphorylation. In addition, Angiotensin II (Ang II), which contributes to hypoxia-dependent PAH, directly increases CF proliferation and collagen expression. The Ang II-induced fibrotic phenotype in CFs also required PKC-dependent downregulation of p38-MAPK (5). Furthermore, cardiomyocyte p38-MAPK activation was previously shown to be a negative regulator of cardiac hypertrophy. Braz and colleagues demonstrated hypertrophy and decreased function in DN-p38-MAPK, DN-MKK3 and DN-MKK6 transgenic mice as well as enhanced hypertrophic responses to aortic banding (3). These authors also found that DN-p38-MAPK and DN-MKK3 mouse hearts exhibited elevated interstitial fibrosis supporting the hypothesis that nicotine-mediated decreases in p38-MAPK activity may be a key signal to promote RV fibrosis. Inhibition of p38-MAPK also enhanced NFAT signaling which is a known mediator of hypertrophy. Nicotine was recently found to increase NFAT activity in neonatal rat cardiomyocytes (16). In contrast, a role for pro-fibrotic activities of p38-MAPK has been proposed based on numerous studies identifying p38-MAPK signaling as a downstream effector of the pro-fibrotic cytokine TGF-β (32). However, some well characterized p38-MAPK inhibitors are also known to inhibit the TGF-β receptor kinase, thus limiting interpretation of these studies (33). Future studies are needed to elucidate the mechanism and role of reduced p38-MAPK activity in nicotine-induced fibroblast proliferation.

Finally, it is noteworthy that nicotine itself is the constituent of cigarette smoke capable of inducing proliferation and fibrotic responses in cardiac fibroblasts. This could have serious implications for chronic nicotine replacement therapy for smoking cessation or recreational use. Future studies will need to determine if nicotine alone can induce the RV fibrosis changes found in CS exposed mice to help identify if continuous nicotine replacement is a safe method of smoking cessation or for recreational use. Further inquiry into the direct effects of nicotine on RV function and fibrosis in relevant animal models and patients will be required.

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Legends:

Figure 1: Assessment of RV and LV function in RA and CS-exposed AKR mice. **A)** Representative images using echocardiography demonstrating TAPSE, tricuspid annulus e', and tricuspid valve E/A, n = 6. **B)** Representative hemodynamic tracings obtained with a Millar pressure catheter placed in the RV and the LV.

Figure 2: Analysis of cardiac fibrosis in RV and LV of RA and CS-exposed mice. RV and LV+S tissue of mice exposed to CS smoke exhibited increased **A**) collagen content (sircol) and **B**) procollagen expression Collagen assay n = 5-7, procollagen expression n = 3-4. * indicates $p < 0.05$ vs room air, Student's t-test. Similar increases in fibrosis were apparent with picrosirius red stained paraffin sections Figure 2C shows representative images of RA and CS-exposed RV and LV. **D**) Significantly increased picrosirius staining in CS-exposed RV but not LV as determined by quantitation of images (see methods). * indicates $p<.05$ vs respective room air exposed tissue using Students T-test, n = 6-7.

Figure 3.Effects of CSE on fibroblast proliferation and role of nAChRs. Cardiac fibroblasts isolated from rats were quiesced for 24 hours, exposed to cigarette smoke extract (CSE) for 24 hours. **A)** CSE dose-dependently increases proliferation (n = 5) and **B**) cell viability (n = 5). **C)** CSE increases proliferation marker PCNA (n = 5). **D)** CSE-induced cell proliferation in cardiac fibroblasts was not attenuated by activation of aldehyde dehydrogenase (Alda-1) or ROS scavenging (NAC) (n = 5). **E)** Mecamylamine (Mec, 20 μM) inhibited CSE-induced fibroblast proliferation (n = 5). Data is normalized to Veh and presented as means ± SEM. * indicates $p < 0.05$ compared to Veh; # indicates $p < 0.05$ compared to CSE alone .

Figure 4. Effect of nicotine on isolated fibroblast proliferation and dependence of α7 nAChR. Nicotine promotes increase (**A**) cell counts (n = 5) and (**B**) collagen content (n = 5) in cardiac fibroblasts through

nAChR activation. **C**) Nicotine also increase procollagen expression (n = 5). **D**) α–BTX (100 nM) blocks nicotine induced proliferation (n = 5). Knock down of α7 nAChR with siRNA (**E**) blocks nicotine-induced proliferation (**F**) (n = 5). Quiescent cells were pretreated with either vehicle, Mec, α–BTX, or α7 nAChR siRNA, followed by vehicle or Nicotine (600 nM) for 24 hrs. Data is normalized to vehicle treatment and presented as means ± SEM. * indicates $p < 0.05$ compared to Veh; # indicates $p < 0.05$ compared to Mec/Vehicle. **G**) There were no differences in expression of α7 nAChR in rat LV and RV tissues (n = 4). **H**) There were no changes in response to nicotine in cardiac fibroblasts isolated from LV or RV or male or female rats. Nicotine significantly increased proliferation in all groups compared to respective untreated groups (indicated by * $p<.05$) (n = 3-4 animals/group performed in triplicate, Three Way ANOVA, SNK post-hoc) **I)** There were no significant changes (T-test) in RV or LV cardiomyocyte contractile function with acute nicotine treatment .

Figure 5: Role of PKC signaling in nicotine-induced fibroblast proliferation: Small molecule inhibitors of PKC-α (100 nM) (**A**) or -δ (3 μM) (**C**), but not PKC-β (**B**) block nicotine-induced CF proliferation (n = 5). Expression of dominant negative PKC-α (**D**) or PKC-δ (**E**) also blocks nicotine-induced CF proliferation (n = 5). Inhibition of PKC-α and -δ block nicotine-induced increased collagen content (**F**) Cells were quiesced in serum-free medium for 24 hours prior to nicotine stimulation. * indicates $p < 0.05$ using two Way ANOVA, SNK post hoc test. **G**) CS smoke exposed mice have increased expression of PKC-α and -δ in RV tissue. A representative blot is shown **H**) Quantitation of data in G (n=4).

Figure 6: Effects of nicotine on phosphorylation of Akt, ERK, and p38-MAPK. **A**) Nicotine treatment for 30 minutes does not change Akt or Erk phosphorylation but does acutely decrease phosphorylated p38-MAPK. Representative blots are shown. **B**) Quantitation of phospho- and total p38-MAPK from A. **C**) Reduced p38-MAPK phosphorylation is blocked by the nAChR antagonist mecamylamine. A representative blot is shown. n = 5. * indicates $p < 0.05$ vs vehicle, unpaired t-test.

Figure 7: Determination of upstream signals regulating nicotine-dependent reduced p38-MAPK phosphorylation. **A**) Inhibition of PKC-α does not reduce p38-MAPK phosphorylation. A representative blot is shown. **B**) Quantitation of results in A. n = 8. **C**) Nicotine-mediated reduction of p38-MAPK phosphorylation is blocked by rottlerin. **D**) Quantitation of results in C. n = 8. * indicates $p < 0.05$ vs control/vehicle group. # indicates $p < 0.05$ from both vehicle and nicotine control groups. Two Way ANOVA, Tukey's test. Any samples in cropped images were run on the same gel with groups irrelevant to the manuscript removed.

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Parameter	Room Air	Cigarette Smoke
PAT (ms)	24.5 ± 2.6	25.9 ± 1.9
LV Ejection Fraction (%)	57.9 ± 7.1	68.7 ± 3.5
Mitral Valve E/A	1.5 ± 0.1	1.4 ± 0.1
Mitral Annulus e' (mm/s)	-23.8 ± 1.9	-19.7 ± 3.0
TAPSE (mm)	1.1 ± 0.1	0.8 ± 0.1*
Tricuspid Valve E/A	0.68 ± 0.05	0.55 ± 0.03*
Tricuspid Annulus e' (mm/s)	-25.1 ± 0.9	-19.4 ± 2.1*
Heart Rate (bpm)	370.2 ± 20.5	396.8 ± 24.7
RVSP (mmHg)	26.3 ± 1.1	27.7 ± 1.7
LVSP (mmHg)	79.6 ± 7.1	74.5 ± 8.3
MPAP (mmHg)	12.0 ± 0.9	13.2 ± 1.3

Table 1: Echocardiographic and in vivo hemodynamic parameters of room air and cigarette smoke exposed mice at 6 weeks. n = 6-12, * indicates $p < 0.05$. PAT: pulmonary acceleration time; TAPSE – tricuspid annular plane systolic excursion; RVSP – RV systolic pressure; LVSP – LV systolic pressure; MPAP – mean PA pressure

Table II – Indices of cardiac hypertrophy and coronary vascular density in Room Air and CS-exposed animals. n = 5-9 for heart body weights. n = 6-8 for histological analysis. * indicates $p < 0.05$

Ventricular Hypertrophy		
	Room Air	**Cigarette Smoke**
Body Wt (g)	31.46 ± 0.91	26.99 ± 0.76*
RV (mg)	40.18 ± 2.31	31.50 ± 2.41*
LVS (mg)	102.62 ± 3.41	90.32 ± 21.2*
RV/LVS	0.39 ± 0.02	0.35 ± 0.03
RV/Body Wt	1.28 ± 0.04	1.19 ± 0.10
LVS/Body Wt	3.30 ± 0.11	3.40 ± 0.06
Coronary Microvessel Density (vessels/mm^2)		
	Room Air	**Cigarette Smoke**
RV	1583.2 ± 78.6	1676.2 ± 112.8
LV	2010.8 ± 82.9	1969.9 ± 120.6

Table III – Indices of alveolar and pulmonary vascular muscularization in RA and CS-exposed animals. n = 4-6 for MLI and n = 3-4 for histological analysis. * indicates $p < 0.05$. MLI – mean linear intercept

Pulmonary Muscularization		
% Vessels	**Room Air**	**Cigarette Smoke**
None	41.6 ± 14.9	45.0 ± 6.7
Partial	36.4 ± 9.4	38.1 ± 3.5
Complete	22.1 ± 6.1	17.0 ± 4.6
Alveolar Space		
	Room Air	**Cigarette Smoke**
MLI	28.9 ± 0.82	32.9 ± 1.18*